THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 240, No. 3, March 1965
Printed in U.S.A.

A Comparison of the Initial Actions of Spleen Deoxyribonuclease and Pancreatic Deoxyribonuclease*

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(Received for publication, September 2, 1964)

The modes of action of several deoxyribonucleases have been studied in varying detail. The manner in which pancreatic deoxyribonuclease degrades deoxyribonucleic acid has been thoroughly analyzed both with regard to the breakdown of secondary structure (1, 2) and to the products formed (3, 4). The activities of several other deoxyribonucleases have been characterized, especially those from *Escherichia coli* (5, 6). A variety of specificities has been observed in which the preference seems to be most directly related to the secondary structure of the substrate and not rigorously to the internucleotide linkage (7).

The use of a well defined, homogeneous substrate, either a synthetic polynucleotide or a DNA prepared from purified bacteriophage, might be expected to lead to the most conclusive elucidation of the mechanisms of DNA degradation by the various DNases. In addition, a very sensitive criterion for assay of the extent of early reaction can be obtained by the use of a viral DNA with measurable biological activity as a substrate (8, 9).

DNase II,[1] isolated from calf spleen, is an endonuclease (10), forming end groups with monoesterified 3′-phosphate group (11). We have studied the effect of this enzyme on the biological activity and sedimentation behavior of homogeneous preparations of DNA from bacteriophage λ. The results definitively support the earlier contention (10, 12) that this enzyme degrades DNA by a "one-hit" process which cleaves both strands of the DNA, either simultaneously or in quick succession, and in close proximity.

EXPERIMENTAL PROCEDURE

Materials

Enzymes—DNase II was prepared from calf spleen by the method of Fredericq and Oth (13).[2] The purification was approximately 500-fold after the third chromatographic fractionation on hydroxylapatite. The solution contained about 2900 units[3] of enzyme per mg of protein. Once recrystallized pancreatic DNase was obtained from Sigma Chemical Company. Just before use, it was diluted in the presence of 0.005 M $MgCl_2$ and 20 μg per ml of bovine serum albumin to the desired concentration.

Bacterial Strains—All strains are derivatives of *E. coli* K12 obtained from Dr. Jean Weigle: 3110, a λ-sensitive strain, was used to grow phage stocks; 3110 (λ) was used to prepare wild type λ-phage by ultraviolet induction and also to plate infective centers in the infectivity assay (see "Methods"); λ-sensitive C600 (14) was used as recipient of DNA infection (see ("Methods").

Bacteriophage—Wild type λ-phage and the mutant b2b5c were gifts of Dr. Jean Weigle. The b mutations are deletions in the DNA (15).

Media—K media (16) was used to grow phage stocks. The other media for growing phage and bacteria are those described by Kaiser (17).

Methods

Growth and Purification of Bacteriophage—Wild type λ-phage was prepared by ultraviolet light induction of the lysogenic strain 3110(λ) (16). The mutant b2b5c was prepared by infecting a logarithmically growing culture of 3110 at 2 to 3 × 10^8 bacteria per ml in K media with about 1 phage per 50 bacteria. Foaming was controlled by the addition of propylene glycol monolaurate, and at 4 to 5 hours, 1 ml of chloroform per 100 ml of culture was added, and aeration was continued for 15 minutes. The yield was 1 to 2 × 10^{11} plaque formers per ml. The lysate was partially purified by several cycles of low and high speed centrifugation. All phages from which DNA was to be prepared were purified by two bandings in a preparative CsCl gradient. The visible bands were collected from a hole punched in the side of the tube, above the pellicle of free DNA which was often present on the bottom.

No treatment with DNase was included during purification of the phage as this resulted in DNA preparations with lowered infectivity and altered sedimentation behavior in alkali. Similar effects have been noticed previously (6).

DNA Preparation—A modification of the phenol technique (18) described by Mandell and Hershey (19) has been employed. To 1 volume of a purified b2b5c phage suspension containing 4 to 6 × 10^{12} plaque formers per ml (in 0.01 M Tris-HCl buffer, pH 7.5, plus 0.3 M NaCl, plus 0.01 M disodium EDTA) is added 1 volume of freshly redistilled, colorless phenol, previously equilibrated with the same buffer solution. The mixture is rocked back and forth just vigorously enough to produce com-

* These studies were supported in part by Grant RG6965 from the United States Public Health Service.

† Predoctoral fellow of the National Science Foundation during part of these studies.

[1] The abbreviation used is: DNase II, spleen deoxyribonuclease (also called acid DNase).

[2] The enzyme was prepared by Steven Goldner.

[3] Units are defined as described by Koerner and Sinsheimer (11) but were measured at 25° rather than 37°.

plete emulsion for 2 minutes. After centrifugation, the aqueous layer is removed by gentle pipetting with a wide bore pipette, and the phenol extraction is repeated once more. The combined phenol layers are extracted with 0.5 volume of buffer, and all aqueous layers are combined and dialyzed for 18 hours against 3 changes of 500 volumes of 0.01 M Tris-HCl, pH 7.5, plus 0.1 M NaCl. Vinyl surgical gloves were worn during manipulation of the dialysis tubing (20). The absorbance ratios, $A_{260\ m\mu}$: $A_{280\ m\mu}$ = 1.9 and $A_{260\ m\mu}$:$A_{230\ m\mu}$ = 2.2, have indicated that all protein and phenol have been removed. The linear monomeric form of λ-DNA was prepared according to Hershey, Burgi, and Ingraham (21). Essentially complete conversion to linear monomers, as determined by band centrifugation, could be achieved at DNA concentrations as high as 60 μg per ml.

Infectivity Assay—DNA from phage λ is infective (9) if certain conditions of bacteria and "helper" phage are met. The modifications to the procedure of Kaiser and Hogness were largely suggested by Dr. Jean Weigle. The P medium of Kaiser was changed to contain 0.5 mg of glycerol per ml instead of glucose. This increased the efficiency of the assay about 5-fold. Wild type λ-phage was used as helper, and the infecting DNA was of the b2b5c genotype in all experiments. Infective centers were plated on *E. coli* 3110(λ). The multiplicity of infection by the wild type helper was 5. So that a reasonable number of plaques could be obtained in each assay, appropriate dilutions were made of the infecting DNA, not the infected complexes. The efficiency of the biological assays reported was 10^{-3} to 10^{-4} infected cells per DNA molecule.

This assay measured the appearance in the progeny phage of two genetic traits carried by the infecting DNA, b5 which controls the immunity of the phage, and c which indicates whether the plaque will be mottled or clear. Phage with this genotype could arise either by replication of the infecting DNA or by a recombination event in which the b5 and c markers (which are closely linked) were incorporated into the genome of the helper phage and subsequently replicated. If the latter process were the primary source of b_5c phage, then these markers themselves would have to be damaged before the assay would detect any inactivation. Kaiser (17), however, found that most often the entire genome of the infecting DNA was recovered intact, indicating that recombination played a minor role in the assay system. When the molecule was broken in two by hydrodynamic shear, the half-molecules were much less infective (about 100 times) than the wholes (17). Consequently, fragments produced by the action of the deoxyribonucleases would not be expected to contribute significantly to the measured infectivity until extensive inactivation had occurred and the intact molecules were a very small fraction of the population. Thus, the inactiva-

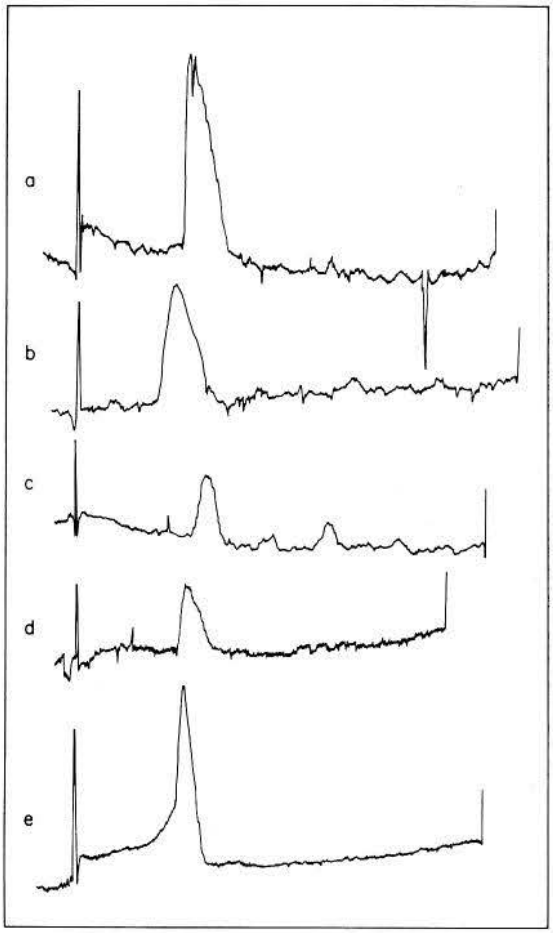

FIG. 2. Band centrifugation patterns of λ-DNA at pH 12. *a*, 4×10^9 wild type λ-phages were layered over alkaline CsCl, pH 12.4, density of 1.253. *b*, λb2b5c DNA Preparation 9; 2 μl of 0.22 M Na_3PO_4 were added to the centrifuge pocket containing 20 μl of DNA at a concentration of 15 μg per ml. The bulk solution was 90% D_2O plus 0.1 M NaCl plus 0.02 M Na_3PO_4, pH 12.2. *c*, b2b5c DNA preparation 6; 20 μl of DNA at a concentration of about 10 μg per ml were layered over alkaline CsCl, pH 12.4, density 1.253. *d*, Preparation 8. Same conditions as *c*, but the concentration was 15 μg per ml. *e*, Preparation 7. Same conditions as *c;* the concentration was about 25 μg per ml. *Meniscus* is at the left.

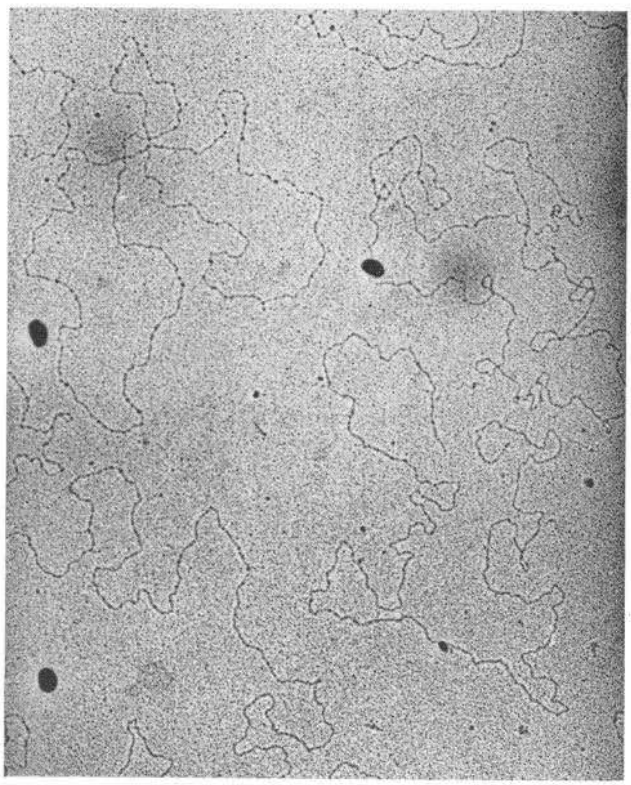

FIG. 1. Electron micrographs prepared by the Kleinschmidt technique; phenol-extracted DNA from phage λb2b5c. Magnification, × 19,000.

tion that is measured at the low levels of inactivation recorded in these experiments is not an effect upon the b5c region specifically, but is an effect upon the whole molecule. For these reasons the term "inactivation of the molecule" seems justified.

Enzyme Assays—With DNase II, the reaction mixture at 37° contained: 0.05 M sodium acetate and 0.01 M sodium citrate buffer, pH 4.7; 0.50 mM $MgSO_4$; 30 to 60 μg per ml of λ-DNA in the linear, monomeric form; and 2 to 30 units per ml of enzyme. The reaction was stopped by chilling and diluting 2-fold with 0.10 M Tris-HCl buffer, pH 9.6, plus 0.06 M $MgSO_4$. The rate of inactivation of biological activity was proportional to the concentration of enzyme.

With pancreatic DNase, the incubation mixture at 37° contained: 1.0 mM Tris-HCl buffer, pH 7.4; 5.0 mM $MgCl_2$, 0.05 M NaCl; 2.0 μg per ml of bovine serum albumin; 30 μg per ml of linear λ-DNA molecules; and 20 μμg per ml of enzyme. The reaction was stopped by 2-fold chilling and dilution of the reaction mixture with 0.02 M Tris-HCl, pH 9.5, plus 6.0 mM disodium EDTA.

Ultracentrifugal Analyses—Sedimentation runs were performed in the Spinco model E ultracentrifuge equipped with an ultraviolet light source and a monochromator. An alternating mask aperture was constructed and used to permit alternate photography of two velocity cells in the same rotor. Thus, centrifuge runs at pH 7 and pH 12 could be compared on the same film. Boundary sedimentation was performed in Kel F centerpieces in 0.1 M NaCl plus 0.01 M Tris-HCl, pH 7, or in 0.02 M Na_3PO_4, pH 12.2, at 35,600 r.p.m. and 20°. (Reconstruction experiments showed the DNA was fully hyperchromic under all conditions used for alkaline centrifugation.) Band centrifugation was performed as outlined by Vinograd *et al.* (22). Film tracings were made on a Joyce-Loebl densitometer. Relative concentrations in the cell were estimated directly from the tracing and these were verified by the reference-wedge method of Robkin, Meselson, and Vinograd (23).

Electron Microscopy—The electron micrographs of phenol-extracted DNA from phage λb2b5c were prepared by a modification of the Kleinschmidt and Zahn technique (24, 25).[4]

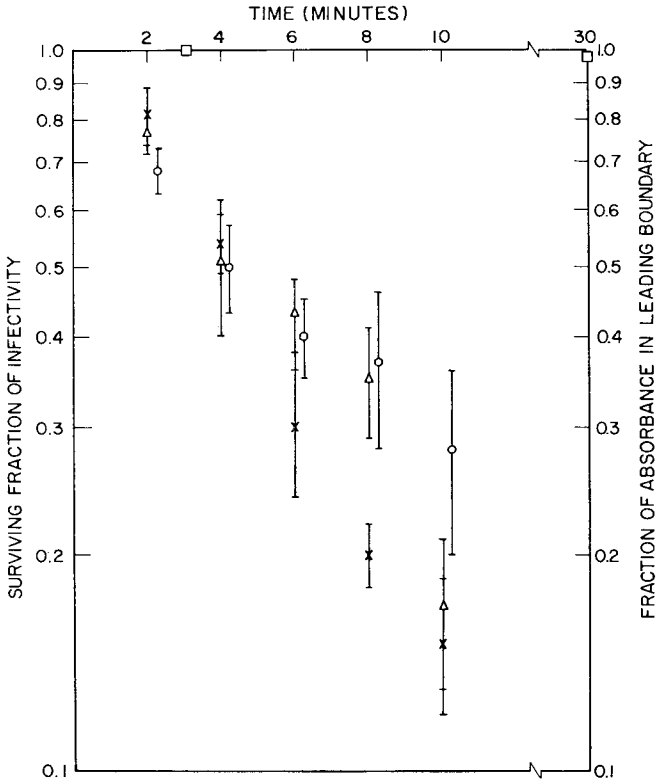

FIG. 3. Degradation of linear λ-DNA by DNase II. The reaction mixture at 37° contained 60 μg per ml of DNA Preparation 6, 3 units per ml of enzyme, 0.05 M sodium acetate, 0.01 M sodium citrate buffer (pH 4.7), and 0.5 mM $MgSO_4$. The reaction was stopped as described under "Methods." ×, surviving fraction of infectivity ± 1 standard deviation measured from three assays; △, fraction of absorbance in leading boundary, pH 7, ± 1 standard deviation usually measured from Frames 6 through 12 which were taken at 6-minute intervals; ○, same, but centrifuged at pH 12; □, reconstruction. An aliquot of the reaction mixture was diluted at zero time with an equal volume of buffer containing 0.10 M Tris-HCl, pH 9.6, and 0.06 M $MgSO_4$. The sample was kept at 25°, and the biological activity was measured at 3 and 30 minutes.

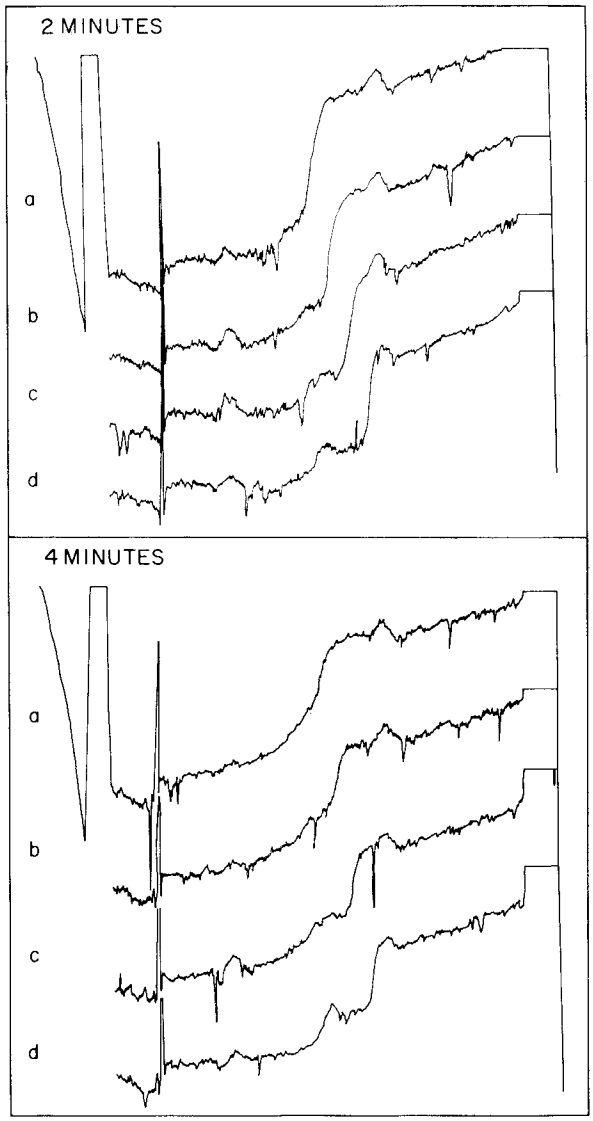

FIG. 4. Boundary centrifugation patterns at pH 12 after 2 and 4 minutes of incubation with DNase II. Reaction conditions as given in Fig. 3. The pictures were taken at 4-minute intervals during centrifugation at 35,600 r.p.m. and 20°.

[4] We would like to thank Clyde A. Hutchison, III, who made the electron micrographs.

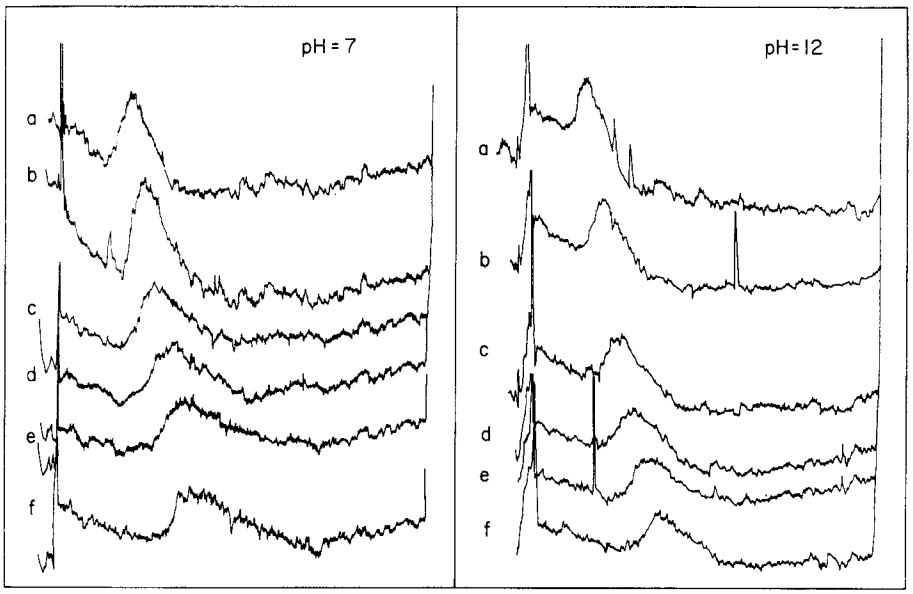

FIG. 5. Band centrifugation at pH 7 and pH 12 of linear λ-DNA degraded by DNase II. Reaction mixture as given in Fig. 3, but 30 μg per ml of DNA Preparation 9 and 1.5 units of enzyme were present. The survival of infectivity was 0.5. After the reaction was stopped, 20 μl of the sample were put in the centerpiece pocket and, for alkaline centrifugation, 2 μl of 0.22 M Na_3PO_4 were added. The bulk solution contained 90% D_2O, 0.1 M NaCl, and either 0.02 M potassium phosphate (pH 7), or 0.02 M Na_3PO_4 (pH 12.2). Pictures were taken at 4-minute intervals, counting *a* as 16 minutes after reaching speed.

RESULTS

Homogeneity of Substrate DNA—The electron micrograph in Fig. 1 shows two rings and one linear λ-DNA b2b5c molecule from an untreated preparation. The average length was 12.1 μ and the standard deviation of the 11 molecules measured was 0.5 μ. No fragments were seen, but many longer molecules (aggregates) (21) were present. The length corresponds to a 26% deletion from the wild type DNA, taking the length of wild type as 16.3 μ (25), which agrees with genetic experiments (15) and other electron microscope observations (26).

The native DNA sedimented as a single species in boundary (21) and band centrifugation after treatment to convert the molecules to the linear form.

Upon alkaline denaturation (27), some DNA preparations which looked homogeneous in band centrifugation at pH 7 gave bands at pH 12 with a varying amount of trailing material. In early experiments in which the phage were treated with pancreatic DNase during purification, the extracted DNA always showed a large amount of this degraded material. However, preparations could be obtained with no significant trailing material. Fig. 2 shows the band sedimentation patterns of four different phenol preparations of DNA from λb2b5c (prepared as described in "Methods") and one pattern in which wild type phage were directly layered over alkaline CsCl, pH 12.4, density 1.253. Only in Pattern e from DNA Preparation 7 is a shoulder of trailing, degraded material obvious. Enzyme experiments were performed only with DNA preparations with which no detectable tail could be seen on band sedimentation at pH 12.

DNase II Degradation: Biological Inactivation and Boundary Sedimentation Analysis—The kinetics of degradation of linear λ-DNA molecules by DNase II are shown in Fig. 3. The residual biological activity, expressed as the fraction of original activity, decreased with first order kinetics for at least two decades. The fractional amount of DNA in the leading boundary at pH 7 and pH 12 decreases in a corresponding fashion for at least one decade. The illustrative densitometer traces in Fig. 4 are from sedimentation runs at pH 12 after 2 and 4 minutes of degradation by DNase II.

Band Sedimentation Analysis—The distribution of DNA in the ultracentrifuge cell at pH 7 and pH 12 after inactivation by DNase II to a survival of infectivity of about 0.5 is shown in Fig. 5. The shapes of the sedimenting bands at pH 7 and pH 12 indicate the same degree of heterogeneity at both pH values in the range from 0.5 to 0.05 surviving infectivity. Table I compares the relative weight average molecular weights calculated from the sedimentation pattern with those calculated from the inactivation of infectivity, assuming inactivation by random degradation of a *single strand polymer*. The expression, derived by Charlesby (28), is

$$\frac{(M_w)_t}{(M_w)_0} = \frac{2(e^{-p} + p - 1)}{p^2}$$

in which $(M_w)_t$ is the weight average molecular weight at time t, and $(M_w)_0$ that before any degradation has occurred. p is the average number of lethal biological hits per initial molecule calculated from the surviving fraction, assuming a Poisson distribution of hits.

To measure the weight average molecular weight from the band sedimentation patterns at pH 7 and pH 12, we subdivided the peaks from three or four tracings from each centrifuge run into 10 fractions of equal area and determined the corrected sedimentation coefficients for the center of area of each fraction. The corresponding molecular weights were calculated by the relation (29),

$$s^0_{20,w} = 0.08\ M^{0.35}$$

for the pH 7 samples. At pH 12, the values of $s_{20,w}$ were first corrected to 1.0 M Na^+ with the use of Studier's[5] expression,

[5] Dr. William Studier very kindly made available to us his ultracentrifuge data prior to its publication.

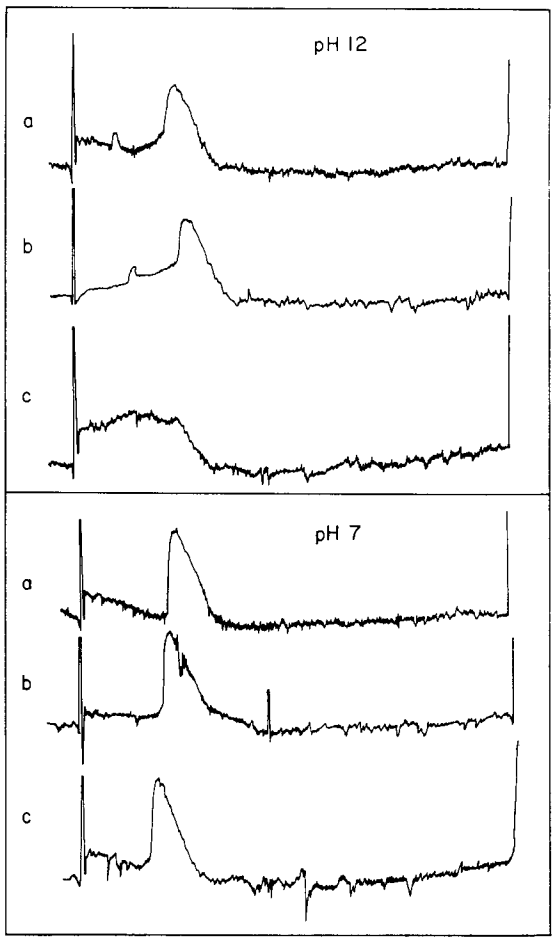

FIG. 6. Band centrifugation patterns at pH 7 and pH 12 of linear λ-DNA degraded by pancreatic DNase. The reaction mixture at 37° contained 1.0 mM Tris-HCl buffer (pH 7.4), 5.0 mM $MgCl_2$, 0.05 M NaCl, 2.0 μg per ml of bovine serum albumin, 30 μg per ml of linear DNA Preparation 9, and 20 μμg per ml of enzyme. The reaction was stopped as described under "Methods." Band centrifugation was performed as given in Fig. 5. *a*, 0.9; *b*, 0.7; and *c*, 0.6 surviving fraction of infectivity, respectively. Pictures taken 24 minutes after reaching speed. *Meniscus* is at the left.

$$(s^0_{20,w})_{1.0\ M} = s^0_{20,w} - 9.25 \log (Na^+)$$

The equation[5]

$$(s^0_{20,w})_{1.0\ M} = 0.0528\ M^{0.400}$$

relating corrected sedimentation coefficient at infinite dilution to molecular weight for denatured DNA in alkali in 1.0 M Na^+ was then used to calculate the molecular weight of each fraction. The sum of the molecular weights divided by 10 provided the weight average molecular weight for that sample. The values reported are averages from two to four such determinations.

Pancreatic DNase Degradation: Biological Inactivation and Band Sedimentation Analysis—After inactivation by pancreatic DNase to 0.9, 0.7, and 0.6 biological survivors, the DNA is distributed in the band-forming cell as shown in Fig. 6. The extent of biological inactivation of the sample in Fig. 6*c* is approximately the same (0.6) as that of the sample shown in Fig. 5 for DNase II. Fig. 7 compares the residual biological activity and the relative amount of undegraded material in the bands at pH 7 and pH 12 as a function of time in the presence of enzyme. From the rate of biological inactivation, the number of lethal hits can be calculated, again assuming a Poisson distribution. Approximating the rate of increase of degraded material at pH 12 by an exponential survival function, the number of single strand breaks as a function of time can be calculated. A comparison with the number of biological hits then indicates that the ratio of single strand breaks to biologically lethal hits is about 4.

DISCUSSION

Previous studies of the effect of DNase II on the secondary structure of native DNA (10, 12) supported a "single hit" mechanism whereby both strands of the duplex were broken at the same level simultaneously. None of the methods used would have detected single chain scissions that might have occurred concurrently. Centrifugation at pH 12 separates the strands of the helix, and the individual polynucleotide chains can be examined. Our results indicate that, during the initial stages of degradation, whenever one strand is broken the complementary strand is also cleaved, causing the original duplex to become two smaller duplexes, each composed of uninterrupted polynucleotide chains. The analysis of the amount of material remaining in the leading boundary after inactivation with DNase II (Fig. 3) indicates that the duplex molecules and single strands are degraded at the same rate. Moreover, this rate is approximately the same as the decay of biological activity, implying that one hit (one break) is sufficient to cause an inactivation. The same result obtains when the method of band centrifugation (22) is used to study the degradation products.

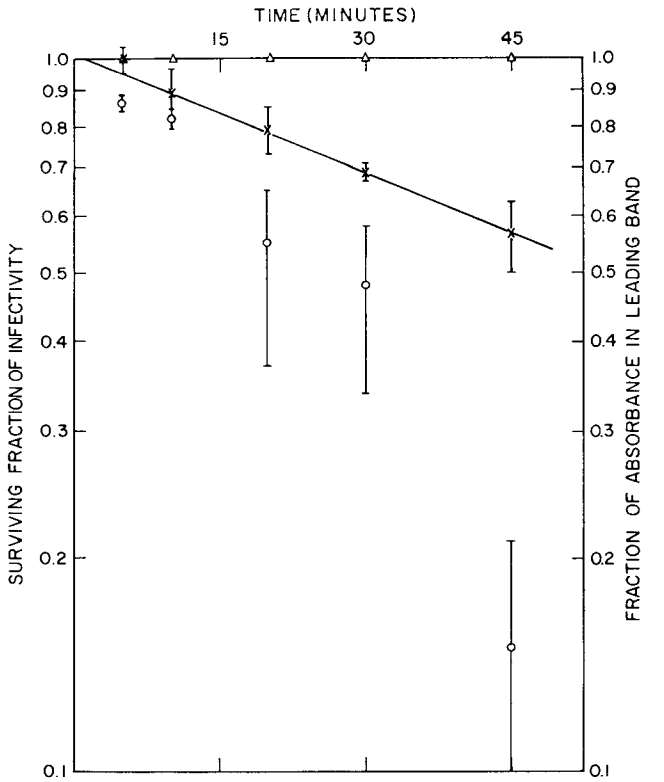

FIG. 7. Inactivation of linear λ-DNA by pancreatic DNase. Digest conditions given in Fig. 6. ×, surviving fraction of infectivity ± 1 standard deviation measured from three assays; ○, fraction of absorbance in leading band, pH 12. The mean and the range of values measured in from 4 to 8 frames, usually the 4th through the 10th, are plotted. △, same, but centrifuged at pH 7.

TABLE I

Relative weight average molecular weights of degradation products of DNase II measured by sedimentation and by biological inactivation

The enzyme assay was performed as in Fig. 5. The absolute and relative weight average molecular weights were calculated as outlined in the text. The number of biological hits was calculated from the inactivation of biological activity assuming a Poisson distribution of hits. $(\bar{M}_w)_0$ is the weight average molecular weight at time zero. $(\bar{M}_w)_t$ is the weight average molecular weight after t minutes of incubation with DNase II. The ratio, $(\bar{M}_w)_t/(\bar{M}_w)_0$, is the relative weight average molecular weight or the weight average degree of polymerization.

Time	Biological		Sedimentation			
			pH 7		pH 12	
	Hits	$\frac{(\bar{M}_w)_t}{(\bar{M}_w)_0}$	$(\bar{M}_w)_t \times 10^{-6}$	$\frac{(\bar{M}_w)_t}{(\bar{M}_w)_0}$	$(\bar{M}_w)_t \times 10^{-6}$	$\frac{(\bar{M}_w)_t}{(\bar{M}_w)_0}$
min						
0	0.0	1.00	27.3	1.00	14.5	1.00
5	0.70	0.82	24.9	0.91	12.5	0.86
10	1.28	0.68	24.2	0.89	9.3	0.64
15	1.63	0.63	20.3	0.74	9.7	0.67
20	1.84	0.59	16.3	0.60	8.0	0.55
45	3.20	0.44	10.9	0.40	4.5	0.31

The experiments performed with pancreatic DNase agree with the accepted mechanism (1, 2). A large number of single strand breaks are necessary before any change in the native structure is apparent by centrifugation at neutral pH. Using very small amounts of enzyme to study the initial stages of degradation, we have been able to show that, on the average, about four single strand breaks per molecule are necessary to inactivate the molecule, biologically. Interestingly, however, the kinetics of biological inactivation are approximately exponential. This implies that any hit has about one chance in four of producing inactivation. This result is similar to those obtained with ^{32}P decay to inactivate phage λ. Using the data from Stent and Fuerst (30) and taking the molecular weight of λ-DNA as 31,000,000, one finds that about one transmutation in seven is lethal to the phage at 4°.[6]

The molecular weight data compiled in Table I emphasizes the difference between degradation by pancreatic DNase and DNase II. Thomas (2) found by titrimetry and light scattering that pancreatic DNase must hydrolyze approximately 200 phosphodiester bonds (on the assumption that the calf thymus DNA used had a weight average molecular weight of 6×10^6), before the weight average molecular weight has decreased by a factor of 2. Our sedimentation data at neutral and alkaline pH indicate that both the duplex and the single strand weight average molecular weights are halved (which corresponds to $p = 2.5$ in Charlesby's equation) at the same time during digestion. At this time, the number of lethal biological hits is also approximately 2.5. The agreement between the decrease in weight average molecular weights as measured by sedimentation (at neutral and alkaline pH) and as calculated from the biological inactivation thus supports the double strand break hypothesis.

All of the enzyme studies reported here were done on DNA preparations which had been found previously by band centrifugation in alkali to contain no significant amount of single strand breaks. The single strand interruptions previously found in the DNA of wild type λ-phage (31) could be in the b2 region which is deleted in the DNA we studied. This region seems to be non-essential to the phage[7] and an interruption might have no biological significance. There are two arguments against this, however. First, a break in such a specific location should give rise to a nonrandom tail of degraded material in boundary sedimentation. That is, two boundaries would be observed. This was not the case (31). Secondly, when wild type λ-phage was layered onto an alkaline CsCl solution in the band-forming centerpiece, no tail of degraded material appeared (Fig. 2). It seems most likely that single strand interruptions are produced during preparation or denaturation of the native DNA.

Although a simultaneous cleavage of both strands by DNase II is apparent, we cannot ascertain from the data whether the breaks occur at precisely the same level in each strand.[8] It seems plausible that the difference in the initial action of pancreatic DNase and DNase II may indicate the presence of two appropriately spaced hydrolytic sites in the latter enzyme. Such an enzyme might play an important role in recombination between DNA duplexes. At later stages of hydrolysis by DNase II, these sites may act independently.

SUMMARY

1. DNA from λ-phage b2b5c can be prepared without single strand interruptions.

2. DNase II degrades λ-DNA by simultaneous cleavage of both polynucleotide chains, at or near the same level.

3. One such cleavage destroys the infectivity of λ-DNA.

4. On the average, approximately four phosphodiester bonds can be hydrolyzed (single strand scissions) by pancreatic DNase in a λ-DNA molecule before its infectivity is lost. However, the kinetics of inactivation imply that a single scission can be lethal.

Acknowledgments—We would like to thank Dr. Jean Weigle for his help in the culture and assay of λ-phage.

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[6] David T. Denhardt, thesis.

[7] J. Weigle, personal communication.

[8] Since this work was completed, an enzyme from *E. coli* (32) has been found to degrade λ-DNA in a manner similar to that observed with DNase II (W. Studier, personal communication).

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